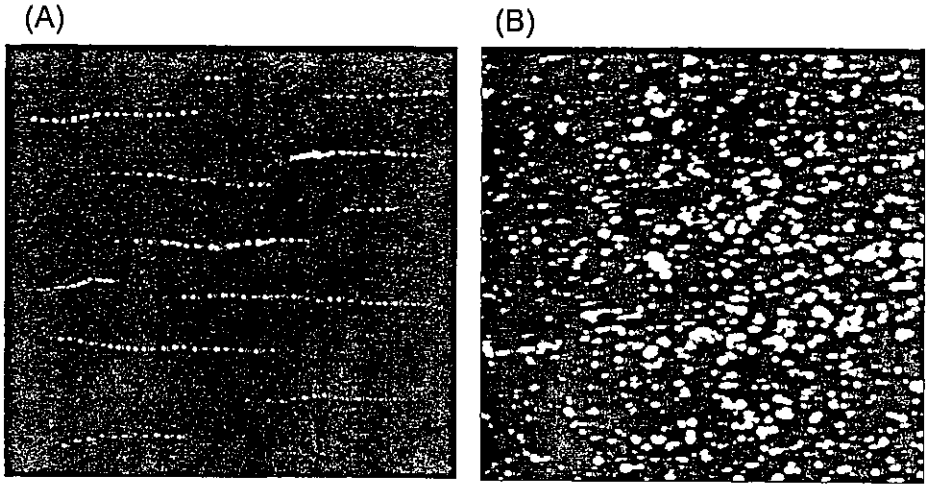

Fig. 1　Observation of (A) rGPIbα-vesicles and (B) rGPIbα-polymerized albumin particles (polyAlb) to the vWf-immobilized surface at the shear rate of 2400s^{-1}. (A) and (B) were superimposed pictures for 3s taken at every 1/6s.

れは，血小板表面の GPIbα と固定化 vWf との相互作用によるものと説明されるが，興味深いことに，rGPIbα 担持小胞体でも血小板と同様に vWf 基板上を転がることが確認された（図 1 (A)）[16]．転がる小胞体の数は高ずり速度の方が多くなり，rGPIbα の特徴が確認できた．また，その転がり速度は小胞体を構成するリン脂質二分子膜の柔軟性（membrane flexibility）と相関した[17]．すなわち，“柔らかい”小胞体では転がり速度は低くなり，“硬い”小胞体では転がり速度は高くなった．これは血流中ではずり応力により“柔らかい”小胞体は変形しやすく vWf 基板と小胞体間の接触面積が増大したために，変形しにくい“硬い”小胞体よりも転がり速度が低下したためと考えられた．このように，rGPIbα の担持により小胞体に血小板機能の一部を付与でき，しかもその機能を制御する方法を見出すことができた．ただし，rGPIbα 担持小胞体は，血小板のように vWf 基板上を転がり続けるのではなく，ある程度転がると脱着する傾向が見られ，これは“柔らかい”小胞体ほど顕著であった．測定前後での小胞体表面の rGPIbα 担持量から，小胞体が vWf 基板を転がっている間に rGPIbα 結合脂質が二分子膜から脱離することが示唆され，脱離し難い rGPIbα 結合脂質を合成して評価を進めている．

他方，低ずり速度の血流下でコラーゲンに直接結合する血小板膜蛋白質の遺伝子組換え体（rGPIaIIa）[18] を結合させた小胞体では，コラーゲン基板を特異的に認識して粘着（停止）する挙動が西谷らによって確認された[19]．この系ではずり速度が高くなるにつれ粘着数は減少するが，rGPIbα と rGPIaIIa を共に担持させた小胞体では低ずり速度から高ずり速度までコラーゲン基板を粘着する[19]．更に，小胞体の内水相に血小板の活性化や凝固系を誘導する因子を内包させておき，出血部位に集積した小胞体がこれらを放出できれば，止血に有効に貢献できるであろう．

4. アルブミン重合体を用いた展開

ヒト血清アルブミンは血漿蛋白質の中で最も多い（5g/dL）蛋白質であり，コロイド浸透圧の調節や栄養物や代謝物などの運搬を担っている．これを利用した微粒子（例えば，アルブミン大凝集体）は生体適合性・生分解性を有するため，既に 1950 年代から静注用製剤として臨床使用されている．数から数十ミクロンの放射化ラベルしたアルブミン大凝集体は，心肺，

脳，肝臓等の血流動態観測用プローブや血管造影剤などに[20]，噴霧乾燥法によって調製したアルブミンマイクロカプセルは超音波診断用増感剤として[21]，アルブミンマイクロスフェアは徐放性の薬物担体として[22]，利用されている．しかし，これらのアルブミン微粒子は，高温や有機溶媒による不可逆的な変性や界面活性剤や架橋剤を用いるため，粒径制御や除去操作が煩雑であった[23]．

我々は，①遺伝子組換えヒト血清アルブミン(rHSA)[24]を単量体としてジスルフィド結合にて重合する方法を用いて，②重合度の制御によりナノスケールからマイクロスケールの粒径制御を可能とし，③水溶液中でのpHと温度の制御にて重合するクリーンな方法により，得られた粒子の表面を親水性とし，④アルブミン変性がほとんどない重合体を得る方法を確立している[25]．

アルブミン重合体は内部が充填された無定形な綿雪のような形態をとっており，例えば出血部位での充填効果が期待できる．表面にrGPIbαを結合させたところ，小胞体のようなvWf基板を転がる挙動は全く認められず，高ずり速度下でも粘着した（図1(B)）．ラテックスビーズにrGPIbαを結合させた系でも同様に粘着することから，担体が重合体である場合と膜構造を持つ小胞体の場合ではrGPIbαの機能発現の仕方が異なることが示唆された．他方，γ線照射にて血小板数を正常値の1/5程度に減少させたマウスにrGPIaIIaを担持させたアルブミン重合体を投与したところ，図2に示すようにコントロール群の出血時間（730 ± 198秒）と比較して，投与量依存的に出血時間の短縮が認められた（例えば，2.4×10^{11} particles/kgでは出血時間は337 ± 46秒）[26]．

さらに減少した残存血小板の凝集を補助するために，粘着して活性化した血小板同士を架橋するフィブリノーゲンを結合させたアルブミン重合体も検討している[27]．活性化血小板の固定化基板を作成し，フィブリノーゲン結合アルブミン重合体を流動させたところ基板上に一様に粘着し，抗GPIIb/IIIa抗体を添加した系やアルブミン重合体のみの系では粘着は認められなかった．更に，血小板数が正常値の1/5程度に調節された血小板減少モデル血液に，添加するフィブリノーゲン結合アルブミン重合体の濃度を増大させると流動血小板の粘着数が増大した

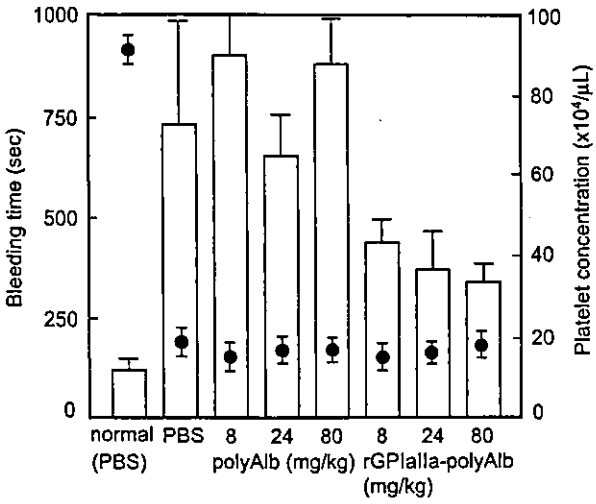

Fig. 2　Effect of the administration of rGPIaIIa-polyAlb on the tail bleeding time (white bar). The administration concentration of rGPIaIIa-polyAlb is 8, 24, and 80 mg/kg respectively, which are values in terms of albumin concentration. •: platelet concentration in the mouse.

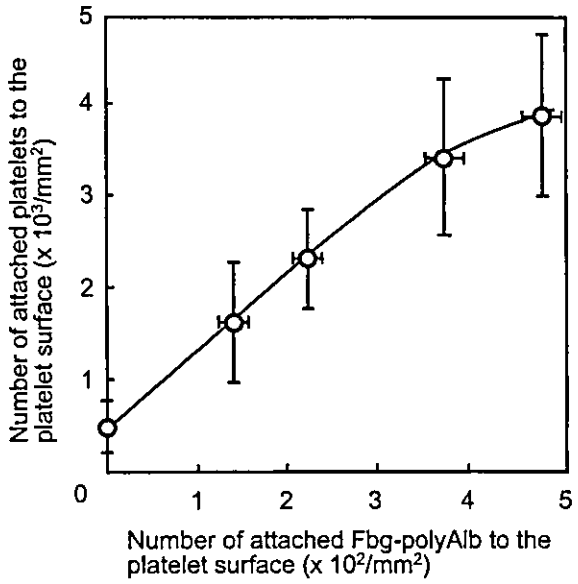

Fig. 3　Effects on the attachment of remaining platelets to the platelet-immobilized surface by the addition of Fbg-polyAlb. The concentrations of Fbg-polyAlb are 0, 1.0×10^{-2}, 2.1×10^{-2}, 3.6×10^{-2}, and 4.7×10^{-2} g/dL.

ことから，フィブリノーゲン結合アルブミン重合体は血小板粘着増強効果を有する微粒子であることが示唆された（図3）．しかし，フィブリノーゲンは不安定であり，しかも現状ではヒト血液由来となる．我々はフィブリノーゲンのγ鎖C末端アミノ酸序列（HHLGGAKQAGDV）を担体に結合させて，フィブリノーゲン機能が安定に発現されることを確かめ[28]，現在，先の認識タンパク質との組合せた系にて検討を進めている．

6. おわりに

血小板代替物の研究は国内外共に浅く，まだ緒に就いたばかりである．血小板減少動物モデルへの投与効果に関する知見も集積されてきた．例えば，GPIbα担持小胞体は主として血管壁側に分布して流れており，出血部位の凝集塊に取り込まれている様子も観測されている（未発表データ）．今後，①血中滞留時間の延長のための担体設計と認識機能発現を高めるための担体設計は一般に相反するので，その工夫が必要となること，②出血部位を認識して止血能を発揮する担体は，血流中で血栓形成が起こらないことを評価する系の確立，③急性時に局所的な止血効果を発現する製剤，あるいは慢性的な全身からの内出血を予防する製剤を設計するための要件の確認など，*in vivo* 評価のフィードバックの段階を踏むことになろう．しかし，現在ではバイオテクノロジーやオプトエレクトロニクスの進歩により血小板の動的な機能に関する多くの情報が短期間に蓄積され，遺伝子組換え蛋白質の大量製造や担体の製剤化技術が飛躍的に進展しているため，近い将来に実用可能な血小板代替物が創製されるであろうし，それは即ち，Drug Local Delivery の基盤技術に貢献することになる．

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Editorial

Propagation of Arterial Thrombi
Local and Remote Contributory Factors

Shinya Goto

It is a common understanding that the rupture of an atheroma in the coronary arteries is the initial event in the onset of arterial thrombosis resulting in myocardial infarction.[1] Ex vivo perfusion experiments using human blood have clearly demonstrated that platelet accumulation occurs immediately when the subendothelial matrix, such as collagen, is exposed to the blood stream.[2] However, initiation of platelet thrombus formation after endothelial disruption resulting in exposure of the subendothelial matrix does not directly represent the onset of symptomatic atherothrombotic diseases, such as myocardial infarction, which were caused by thrombotic arterial occlusion. For example, coronary intervention, while causing damage to the endothelium, does not, in most cases, result in any symptomatic myocardial ischemia. Moreover, recent advances in clinical imaging techniques, such as intracoronary ultrasonography, have revealed a much higher incidence of atheroma rupture than of symptomatic atherothrombotic coronary artery diseases, including myocardial infarction and unstable angina pectoris.[3,4] These observations suggest the contribution of propagating factors for thrombus growth, besides exposure of the subendothelial matrix due to endothelial disruption, in the onset of symptomatic arterial thrombotic diseases.

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In this issue of *Arteriosclerosis, Thrombosis, and Vascular Biology*, Yamashita et al have elegantly demonstrated in experimental studies the importance of 2 factors in the formation of arterial occlusive thrombi, namely increased vascular wall thrombogenicity induced by the accumulation of tissue factor and reduction of the total arterial blood flow by increased vascular resistance.[5] The former explains the difference between the onset of myocardial infarction induced by the rupture of an inflamed tissue factor–rich atheromatous plaque[1] and the asymptomatic limited-size thrombus formation initiated by coronary intervention. Blood flow reduction, induced either by local blood flow disturbance after rupture of an atheroma or by increased microvascular resistance[6,7] also plays an important role in the propagation of arterial thrombi. In addition, embolization of microvessels by platelet-rich thrombi,[8] as well as the microvessel contraction induced by bioactive substances released from activated platelets[9] such as thromboxane A_2, may play some roles in the reduction of arterial blood flow.

von Willebrand factor, along with its putative platelet receptor GPIbα, plays a crucial role in the initiation of platelet thrombus formation (Figure).[10] Then the stimulation of various platelet surface receptors, including integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa),[11] $\alpha_v\beta_3$ (vitronectin receptor),[12] and catecholamine receptor,[13] as well as ADP receptors ($P2Y_{12}$ and $P2Y_1$)[14–16] and others, were involved in the growth of platelet thrombi. Even with those stimulations, platelet thrombi could not grow large enough to cause arterial occlusion by themselves without contribution of fibrin formation. Tissue factor, which is known to initiate coagulation cascade,[1] plays a role in fibrin formation around platelet thrombi to cause so called stable mixed thrombi. These contributing effects of tissue factor have been clearly demonstrated by Falati et al with the use of intravital microscopy.[17] There still is the discussion on the origin of tissue factor incorporated in the arterial occlusive thrombi. Those present in the vascular wall may play an important role as demonstrated by Yamashita et al,[5] although tissue factor in circulating blood, either in a soluble form or in association with membrane of microparticle, may also be involved.[18]

Until now, the beneficial effects of antithrombotic therapy were supposed to be mediated only by their inhibitory effects on the initiation and growth of thrombi at the site of occlusive thrombus as formation takes place. The experimental results reported by Yamashita et al may suggest other possible mechanisms of action of antiplatelet drugs in the prevention of occlusive thrombus formation, such as the role of aspirin in reducing the vasoconstriction mediated by the inhibition of thromboxane A_2 production,[19] the role of anti-GPIIb/IIIa in the prevention of distal embolization,[6,20] or the effects of clopidogrel in the prevention of release of vasoactive substances from activated platelets.[21] According to the experimental results published by Yamashita et al, it might be reasonable to suppose that the inhibition of the accumulation of thrombogenic substances in the vascular wall, as well as the preservation of microvessel function, may be the new targets for the prevention of symptomatic arterial thrombotic diseases.

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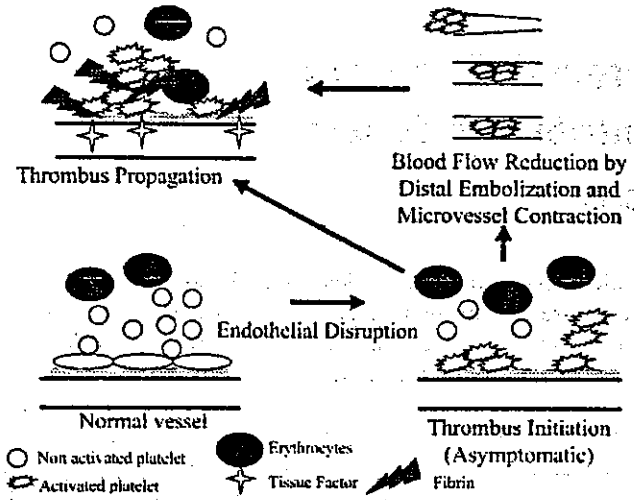

Mechanism of thrombus initiation and thrombus propagation. Endothelial damage induced by any cause, such as atheroma rupture, erosion, or vascular interventional treatment, initiates platelet accumulation and thrombus formation; however, most of the thrombi do not grow large enough to cause clinical symptoms. The size of the thrombus is augmented when the blood flow velocity is also reduced because of increased vascular resistance. Distal embolization, along with microvascular contraction caused by bioactive materials released from activated platelets, such as thromboxane A_2, may play important roles in increasing the vascular resistance. Accumulation of tissue factor in the vascular wall, mostly originating from the inflammatory cells migrated into the vascular wall, along with the fibrin deposition initiated by it, also enhances the propagation of thrombi. Blood flow reduction by small vessel embolization and increased vascular wall thrombogenicity mediated by tissue factor accumulation are presumed to play crucial roles in the formation of arterial occlusive thrombi causing symptomatic diseases.

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Platelets, after Exposure to a High Shear Stress, Induce IL-10-Producing, Mature Dendritic Cells In Vitro[1]

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There is evidence for immune system involvement in atherogenesis. In the present study the effect of platelets on dendritic cells (DC), an important immunologic regulator, was examined in vitro. Platelet-rich plasma, after exposure to shear stress, was added to human monocyte-derived immature DC, which were then examined for surface Ag expression, allogeneic T lymphocyte stimulatory activity, and cytokine production. After exposure, the number of anti-CD40 ligand (anti-CD40L) and anti-P-selectin IgG molecules bound per platelet was increased. These activated platelets induced DC maturation, as revealed by significant up-regulation of CD83, CD80, and CD86 Ags. The addition of platelets in the presence of IFN-γ plus LPS significantly enhanced IL-10 production from immature DC. After platelet addition, mature DC provoked a significant proliferation of allogeneic naive T lymphocytes. These activated T cells showed lower IFN-γ production than those stimulated by LPS- and IFN-γ-treated DC. CD40L on the platelet surface was not involved in maturation of DC, as mAb to CD40L failed to block maturation. The effect of platelets was observed even if platelets and DC were separated using large pore-sized membranes or when platelets were depleted from plasma by centrifugation. Furthermore, it was abrogated after the depletion of protein fraction. Thus, soluble protein factors excreted from activated platelets contribute to IL-10-producing DC maturation. *The Journal of Immunology,* **2004, 172: 5297–5303.**

The role of cross-talk between platelets and leukocytes in the local inflammatory response, particularly with regard to involvement in the chronic inflammatory aspect of atherosclerosis, has long been discussed (1). Several murine studies revealed that immune systems were tightly involved in the course of atherogenesis (2). Among them, recruitment of monocytes was shown to be important for the formation of atherosclerosis (3). Experiments using IFN-γ receptor-deficient, Apoe$^{-/-}$ mice proved that Th1-skewed immune reactions participate in the formation of atherosclerosis (4). Th1 cells were dominant in early phases of atherosclerosis (5). Dendritic cells (DC),[3] the most potent APC, initiate innate and acquired immune responses (6). After treatment with LPS plus cytokines, DC secrete large amounts of IL-12 and IL-18, both of which are expressed in atherosclerosis lesions, which augment IFN-γ production (7, 8). Although their developmental pathways are quite complex, one DC population differentiates directly from monocytes (monocyte-derived DC) and migrates into endothelial cells using specific chemokine receptors (9), which suppose its tight involvement in the plaque formation similar to monocytes or macrophages. Recent investigations have revealed the presence of DCs at atherosclerosis-related lesions, especially at sites prone to rupture (10, 11).

Whether activated platelets attached to an atherosclerotic lesion influence plaque formation has not yet been elucidated (12). By releasing adhesive ligands, platelet-derived growth factor, or β-amyloid precursor proteins, platelets can provoke activation of APC (1, 13). Platelets express CD40 ligand (CD40L) after stimulation by thrombin (14). The role of CD40L in the progression of atheromatous plaque is of particular interest, as many recent reports indicate the presence of CD40 on the surface of atheroma-related cells, such as endothelial cells, macrophages, and smooth muscle cells (15). As the CD40-CD40L interaction is important in the progression of atherosclerosis (16) as well as plaque rupture mediated by increased matrix metalloprotease production from macrophages and T cells (17), interruption of CD40L binding to CD40 receptors or inhibition of CD40L expression on platelets may be a new therapeutic strategy to prevent progression of atherosclerosis and atheroma rupture. The function of DC is mediated by CD40 ligation by CD40L (18). Recently, Hilf et al. (19) reported that human platelets inhibited DC activation in the presence of heat shock protein and suggested that a low concentration of platelets was a reason for failed DC activation.

We have focused on the direct interaction between platelets and DC. Several groups have proposed that the mechanism of in vivo platelet activation might not be the same as that of platelet activation induced by chemical agonists such as thrombin (20). Indeed, multiple receptor-ligand interactions, including many plasma

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[3] Abbreviations used in this paper: DC, dendritic cell; CBA, cytometric bead array; CD40L, CD40 ligand; MNC, mononuclear cell; UCB, umbilical cord blood; VWF, von Willebrand factor.

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and matrix proteins with their corresponding platelet surface receptors, are involved in the process of platelet activation after interaction with subendothelial matrix exposed at sites of endothelial damages (21, 22). Von Willebrand factor (VWF) and its interaction with platelet receptors, gpIb/IX and gpIIb/IIIa, are particularly important, especially at sites exposed to high shear stress (12, 20, 21, 23). Previous reports clearly demonstrated the appearance of immune-related proteins, such as CD40L and P-selectin, on the surface of platelets after exposure to a high shear stress (23, 24). In the present study we clarified the role of activated platelets after exposure to high shear stress on maturation and cytokine production of monocyte-derived DC. DC are currently recognized not only as immunogenic but also as tolerogenic when they are in immature or semimature stages (25). These opposite paths are determined by the release of proinflammatory cytokines, such as IL-12 or IL-10. Mature DC in general, through the production of IL-12, are inducers of Th1 immune effectors, whereas immature or semimature DC induce Th2 immune effectors (26). Thus, key cytokines (IFN-γ for Th1, IL-5 for Th2) which are produced from naive T lymphocytes after stimulation with platelet-activated DC were examined.

Materials and Methods

Blood sample preparation and shear-induced platelet activation

Blood was collected from 10 healthy adult donors who abstained from the use of drugs known to interfere with platelet function, such as NSAIDs. Blood was immediately treated with trisodium citrate or with the specific thrombin inhibitor PPACK to keep physiologic concentrations of divalent cations. Platelet-rich plasma was separated by centrifugation at 100 × *g* for 15 min. Platelets in 400 μl of platelet-rich plasma were exposed to a high shear rate of 10,800 s^{-1} for 6 min in an optically modified cone-plate viscometer (20), the shear rate previously shown to induce VWF-gpIb/IX-mediated platelet activation (20, 23, 24). Shear-induced platelet aggregation was blocked by the specific anti-gpIIb/IIIa agent tirofiban, enabling detection of CD40L and P-selectin molecules expressed on single platelets as we previously reported (24). Umbilical cord blood (UCB) was obtained with written informed consent after approval by the human subjects committee of Tokai University Hospital.

Effects on DC maturation

Mononuclear cells (MNCs) were separated from blood samples by Ficoll-Hypaque density (1.077 g/dl) gradient. $CD14^+$ cells were isolated from MNCs using MACS CD14 immunomagnetic beads (Miltenyi Biotec, Gladbach, Germany), followed by a MACS-positive selection column (Miltenyi Biotec). DC were induced from $CD14^+$ cells by culture for 7 days in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FCS (Life Technologies, Gaithersburg, MD) and supplemented with GM-CSF (100 ng/ml; a gift from Kirin Brewery, Maebashi, Japan), and IL-4 (10 ng/ml; a gift from Ono Pharmaceutical, Osaka, Japan)

DC were then incubated with platelet samples. In all experiments platelets were prepared from 10 ml of heparinized human blood and adjusted to a 200-μl final volume. Addition of 100 and 10 μl corresponded to 1000 and 100 for the platelet to DC ratio, respectively. Positive and negative controls for DC maturation were obtained by culture in the presence or the absence of LPS (100 ng/ml; Sigma-Aldrich) and IFN-γ (1000 U/ml; Shionogi, Osaka, Japan). The level of DC maturation was checked by double-immunofluorescence staining using FITC-anti-CD1a mAb (DAKO, Glostrup, Denmark)/PE-anti-CD83 mAb (Immunotech, Marseilles, France), and FITC-anti-CD80 mAb (Immunotech)/PE-anti-CD86 mAb (Immunotech). In additional experiments, anti-CD40L mAb (Ancell, Bayport, MN) was added together with activated platelets to test the role of CD40L on DC maturation. The efficacy of mAb was confirmed by inhibition of the maturation effect by CD40L transfectant (CD40L; provided from Dr. Schultze, Dana-Farber Cancer Institute, Boston, MA). In the next experiment, platelets were placed in the chamber of a Transwell insert with high density-sized pores (BD Biosciences, San Jose, CA), which prevented direct contact between platelets and DC. Plasma, after depletion of shear-stressed platelets by centrifugation (10,800 s^{-1}, 6 min), or plasma without any treatment was added. In the next experiment, protein components among these plasma samples, after depletion of shear-stressed platelets, were depleted by ethanol precipitation, then added to immature DC.

Effects of platelets after shearing on production of cytokines from DC

Immature DC were washed extensively and replated at a density of 10^5/96-well, flat-bottom plates with 10% FCS containing RPMI 1640. These DC were cultured for 48 h with or without the addition of platelets exposed to a high shear rate in the presence or the absence of LPS (10 μg/ml) plus IFN-γ (1000 U/ml). The culture supernatant was harvested and examined for the quantity of IL-10 and IL-12p70 by ELISA (Immunotech)

Effects of sheared and nonsheared platelets on the proliferation of allogeneic naive T lymphocytes in response to DC and on cytokine production

UCB-MNCs, depleted of $CD14^+$ cells by MACS column separation, were added at 6 × 10^4/well to 96-well, round-bottom plates as responders. Immature DC, DC matured by LPS and IFN-γ, and DC incubated with platelets exposed or not exposed to a high shear stress were serially diluted after radiation (15 Gy) and added as stimulators. The cells were cultured for 6 days in RPMI 1640 with 10% pooled human sera, then counted for their proliferative activity by the [^{3}H]thymidine uptake test. Simultaneously, the culture supernatant was harvested from the cultured wells of immature, LPS- and IFN-γ-treated DC, or DC incubated with 100 μl of platelets as stimulators and examined for the content of cytokines (IFN-γ and IL-5) using a cytometric bead array (CBA) kit (BD Biosciences).

Naive T cells after incubation with DC were cultured for another 4 days in the presence of IL-2 (20 U/ml; Shionogi), incubated with 20 μg/ml PMA (Sigma-Aldrich) and 10 μg/ml ionomycin (Sigma-Aldrich) for 5 h in the presence of brefeldin A (Sigma-Aldrich) and permeabilized, and the intracellular expression of IFN-γ and IL-4 was examined using FITC-IFN-γ and PE-IL-4 (BD Biosciences) by flow cytometry. Also, the cultured cells were extensively washed and replated at a density of 10^5/200 μl in the presence of PMA/ionomycin, and their supernatant was harvested 16 h later to examine the cytokine (IFN-γ, IL-4, IL-5, and IL-10) concentrations using a CBA kit.

Statistical analysis

All numerical data are expressed as the mean ± SD unless otherwise specified. The difference between two groups of data was tested by Student's paired *t* test or unpaired *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

Platelet surface expression of CD40L and P-selectin after exposure to a hgh shear rate

The numbers of platelet-expressing CD40L and P-selectin as well as the mean number of CD40L and P-selectin molecules expressed on the platelet surface were calculated according to a previously described procedure (20, 24). Quantitative analysis revealed that the mean number of anti-CD40L and anti-P-selectin IgG molecules bound per platelet increased from 17 ± 156 and 34 ± 148/platelet before to 388 ± 331 and 1122 ± 554/platelet after shearing (both $p < 0.01$). These results represent the appearance of CD40L and P-selectin, not representing the nonspecific increase in nonspecific IgG binding to activated platelets, because the number of isotype-matched negative control Ab against thyroglobulin (T-γ) did not increase even after exposure to the same shear stress (24). Shear-induced CD40L and P-selectin expression was mediated by VWF-gpIb/IX interaction because mAb blocking VWF-gpIb/IX interaction, both anti-gpIb/IX and VWF, completely inhibited shear-induced platelet surface translocation of those molecules (24).

Effects of platelets on DC maturation

As shown in Fig. 1, immature DC started to express cell surface markers specific for matured DC, CD80, CD83, and CD86 after being cultured with platelets exposed to a high shear rate. Furthermore, the enhancing effect was dose dependent. However, no maturation was observed when DCs were incubated with platelets not exposed to shear. Stimulation with LPS and IFN-γ significantly enhanced surface expression of CD83, CD80, and CD86 compared

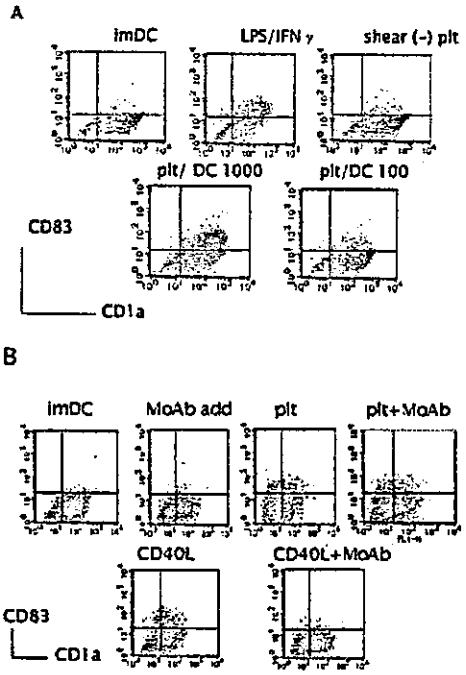

FIGURE 1. Profiles of DC maturation markers. *A*, DC were induced from $CD14^+$ monocytes using GM-CSF and IL-4, then cultured as immature DC (imDC), matured with LPS and IFN-γ, or added with platelets not exposed (shear (−) plt) or exposed to high shear stress in serial dilutions (plt/DC ratio, 1000 and 100) for 48 h. Maturation of DC was detected by CD1a, CD83, CD80, and CD86 expression by double-immunofluorescence staining. *B*, Immature DC were incubated with platelets (plt/DC ratio, 1000) or CD40L transfectant in the absence or the presence of anti-CD40L mAb (20 μg/ml) for 48 h. Maturation of DC was detected by CD1a, CD83, CD80, and CD86 expression by double-immunofluorescence staining.

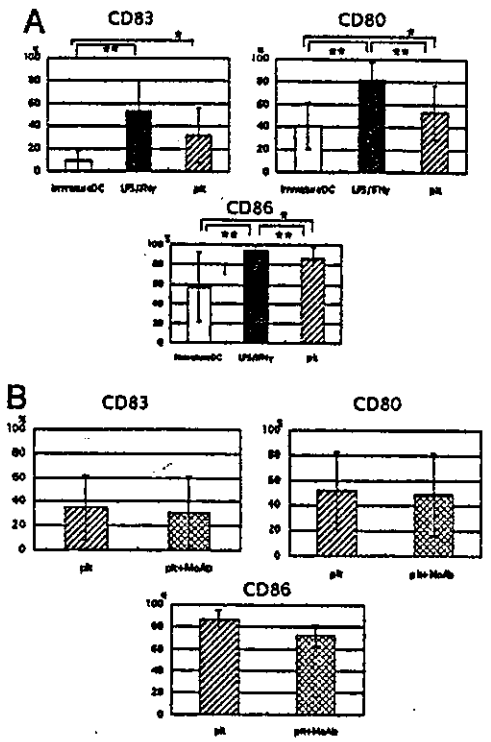

FIGURE 2. Difference in frequencies in surface markers specific for DC maturation and effect of anti-CD40L mAb. *A*, DC were generated as described in Fig. 1. Differences in each marker (CD80, CD83, and CD86) among immature DC, LPS- and IFN-γ-treated DC, and DC incubated with platelets (platelet (plt)/DC ratio, 1000) after exposure to high shear stress were compared. *B*, Anti-CD40L mAb (20 μg/ml) was added together with plt (plt/DC ratio, 1000). The mean ± SD of 10 experiments (samples) are presented, and statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$).

with no stimulation (Figs. 1 and 2; $p < 0.01$). Also, a statistically significant difference ($p < 0.05$) in Ag (CD83, CD80, and CD86) expression was found between DC without and with platelet addition (platelet/DC ratio, 1000). Unexpectedly, mAb to CD40L did not significantly neutralize the enhancing effect of platelet-induced Ags (Fig. 1*B*), whereas it prevented an up-regulation of CD83, CD80, and CD86 Ags by CD40L expressed feeder cells. As shown in Fig. 3*A*, platelets could still up-regulate the expression of CD86 Ag, even if cell-to-cell contact was avoided using the Transwell insert. Furthermore, plasma, after depletion of shear-stressed platelets, could still show maturation-enhancing effects, as shown in Fig. 3*B*. Plasma without shearing did not have such a maturation effect. As shown in Fig. 4, these DC maturation effects were completely abrogated after depletion of the protein fraction.

Effects of platelets on IL-10 and IL-12 production

Untreated DC did not significantly produce either IL-10 or IL-12, as revealed by ODs showing almost the lowest level of detection. As shown in Fig. 5*A*, addition of platelets slightly induced IL-10 production, whereas no IL-12 production was observed. In the presence of LPS and IFN-γ, platelet addition did not influence IL-12 production, but significantly enhanced IL-10 production (Fig. 5*B*). As a result, DC exposed with LPS and IFN-γ plus platelets showed comparable levels of IL-12 and IL-10 production. CD40L mAb did not affect the enhancement of IL-10 production by activated platelets (data not shown).

Effects of platelets on proliferation of allo-T lymphocytes and cytokine production from T lymphocytes

As shown in Fig. 6, the magnitude of allogeneic naive T cell proliferation was highest when they were stimulated with DC matured by LPS and IFN-γ. Although the magnitude was not as prominent, DC after platelet addition (platelet/DC ratio, 1000) significantly promoted allo-naive T cell proliferation. No significant proliferation was observed when T cells were stimulated with DC cocultured with nonsheared platelets or immature DC. Control platelets did not show any effect of proliferation (data not shown). As shown in Fig. 6, none of the DC samples provoked syngeneic T lymphocyte proliferation.

As shown in Fig. 7, T lymphocytes after incubation with LPS- and IFN-γ-matured DC produced IFN-γ, whereas no significant production was obtained when they were cocultured with platelet-matured DC or immature DC. IL-5, one of the main Th2 cytokines, was produced in low concentrations from mixed cultures with immature DC, LPS and IFN-γ, or platelet-matured DC. As shown in Fig. 8, $CD4^+$ naive T lymphocytes after incubation with platelet-matured DC exhibited much lower intracellular expression of IFN-γ than those stimulated with LPS and IFN-γ/DC. However, the levels of the other cytokines (IL-4, IL-5, and IL-10) were equivalent in T cells preactivated with LPS and IFN-γ/DC and those with shear-stressed platelets.

Discussion

The roles of platelet-derived molecules, including platelet-derived growth factor (1), integrins (1), P-selectin (27), and platelet-derived microparticles (28), in regulating local inflammatory response have been investigated. We have demonstrated that the proteins known to interact with leukocytes, P-selectin and CD40L, were surface translocated by high shear rate conditions, even in the

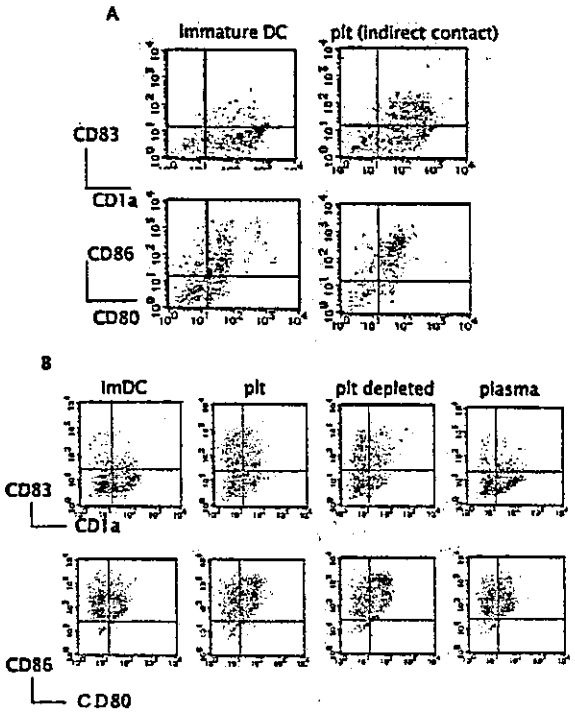

FIGURE 3. Indirect contact between DC and platelets after high shear stress. *A*, Platelets after high shear stress were placed in the chamber of a Transwell insert with high density-sized pores, which prevented direct contact between platelets (plt) and DC. Under the chamber, DC were cultured for 48 h, and their surface marker expression was compared before and after the addition of platelets. *B*, Plasma before and after depletion of shear-stressed platelets or plasma without any treatment was added to immature DC, and their surface marker expression was analyzed.

absence of any known platelet-activating agent, such as ADP or thrombin. P-selectin, but not CD40L, plays a role in the physical interaction between flowing leukocytes and collagen-adherent platelets (29). By contrast, CD40L plays functionally important roles in the maturation of DC, resulting in specific Ag expression, allo-T cell proliferation, and cytokine production (30). These results suggested that platelets play important roles in the regulation of a local inflammatory response at sites exposed to high shear stress by trapping monocytes that can be transformed to DC and by inducing their maturation through stimulation of the CD40 receptor by surface-translocated CD40L. Although CD40L surface translocation can be induced by platelet activation by soluble agonists such as thrombin (14, 31), these are not likely to play important roles in vivo, because high concentrations of soluble agonist are not likely to exist in vivo, especially in the presence of blood flow. VWF-gpIb/IX-mediated platelet CD40L expression is more likely to occur at sites exposed to arterial blood flow conditions generating high shear rates, as animal experiments clearly demonstrate the crucial role of this interaction in arterial thrombosis (32). Similar VWF-mediated CD40L expression is likely to occur on platelets interacting with exposed subendothelial matrix, such as collagen, because VWF has specific collagen binding sites, and the VWF-gpIb/IX interaction plays a crucial role in platelet thrombus formation on collagen (21, 33).

We clearly demonstrate that immature DC become mature when they are incubated with platelets after exposure to high shear stress. DC matured with platelets expressed all CD83, CD80, and CD86 Ags at a significantly high level compared with those without maturation. IL-12 production from immature DC was not enhanced by the addition of shear stress-activated platelets. IL-10

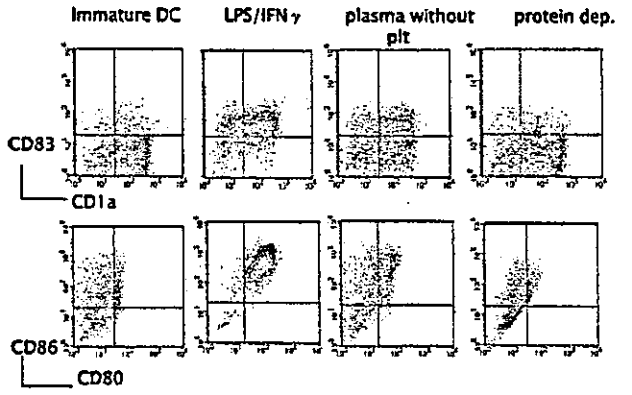

FIGURE 4. Effect of protein depletion. Immature DC were left untreated or were incubated with LPS and IFN-γ, with plasma after depletion of shear-stressed platelets (plasma without plt), or with protein-deprived solution from these treated plasma (protein dep.) Maturation of DC was detected by CD1a, CD83, CD80, and CD86 expression by double-immunofluorescence staining.

production was promoted by platelet addition in the absence and the presence of LPS and IFN-γ. As a result, naive T cells after stimulation with platelet-matured DC did not acquire the ability to produce the Th1 cytokine, IFN-γ, whereas they maintained IL-5 production. Gatti et al. (34) applied fixed activated platelets to Langerhans cells, which were activated by CD40L on platelets. In contrast, our results have shown that CD40-CD40L interaction did not play an important role in maturation of DC, as revealed by mAb blocking tests. Henn et al. (31) reported that CD40L expressed on the platelet surface is transient and is cleaved to a soluble form after stimulation by soluble agonists, although no soluble CD40L Ag was detected in culture supernatant of DC after exposure to high shear stress by ELISA (data not shown). Hilf et al. (19) recently reported that thrombin-activated platelets expressed heat shock protein (gp96) receptors and down-regulated Gp96-mediated DC maturation. They also observed an effect of platelets on DC without Gp96 maturation, but in contrast to our results, neither a change in surface Ags nor enhanced IL-10 was observed. The ratio of platelets to DC (20:1) was far less than that in our experiment (100:1 to 1000:1), which might be a reason why

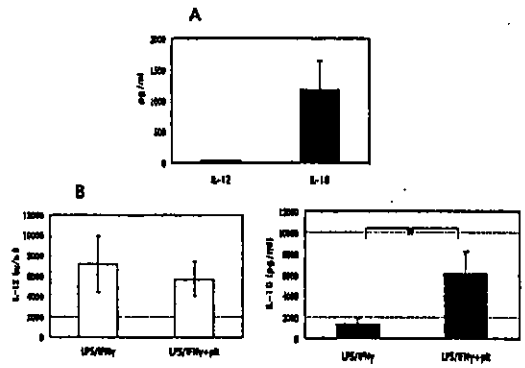

FIGURE 5. Production of cytokines from DC. Immature DC were washed extensively and replated at a density of 10^5/96-well, flat-bottom plate with 10% FCS containing RPMI 1640. These DC were cultured for 48 h with or without the addition of platelets exposed to high shear in the absence (*A*) or the presence (*B*) of LPS (10 μg/ml) plus IFN-γ (1000 U/ml). The culture supernatant was harvested and examined for the quantities of IL-10 and IL-12p70 by ELISA. The mean ± SD of 10 experiments are shown, and statistically significant differences are indicated (*, $p < 0.05$). Although not shown in this figure, DC with no treatment were not capable of cytokine production.

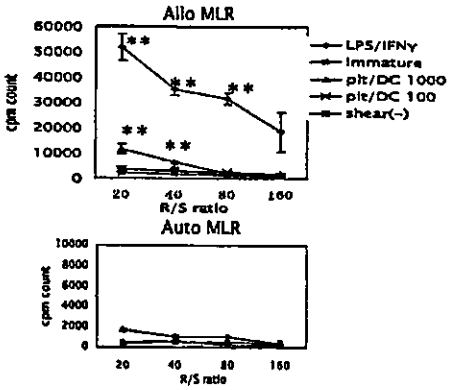

FIGURE 6. Allogenic and syngeneic T cell stimulation. Allogeneic UCB-MNCs (Allo-MLR) or syngeneic adult-MNCs (AutoMLR), depleted of $CD14^+$ cells by MACS column separation, were added at 6×10^4/well to a 96-well, round-bottom plate as responders. Immature DC, DC matured by LPS and IFN-γ, and DC incubated with platelets exposed or not exposed to high shear were serially diluted after radiation (15 Gy) and added as stimulators. The cells were cultured for 6 days in RPMI 1640 with 10% pooled human sera, then proliferative activity was determined by the [^{3}H]thymidine uptake test. Representative data are shown as the mean ± SD of triplicate wells. **, $p < 0.01$ compared with immature DC.

discrepant results were obtained. The functionally important roles of platelet-expressed CD40L in local inflammation and progression of atherosclerosis suggest that drugs inhibiting platelet CD40L translocation might prevent atherosclerosis. However, contrary to our initial hypothesis, our results clearly prove that soluble factors, other than CD40L, excreted from platelets after shear stress, including many of the candidate materials released from α granule, such as soluble P-selectin (27), and those released from dense granule, such as ADP (35), might contribute to maturation of DC. To differentiate whether the soluble factor made by platelets is lipid or protein, protein fraction was depleted by simple methods of ethanol precipitation. This resulted in the total disappearance of maturation effects or promotion of IL-10 production in the presence of LPS and IFN-γ (data not shown). Activated platelets make abundant lysophosphatidic acid (36–38), a bioactive lipid mediator that can enhance the secretion of IL-10 from DC (39). The addition of diacylglycerol pyrophosphate (8:0), which is a selective antagonist of lysophosphatidic acid (37), did not produce any neutralization effect.

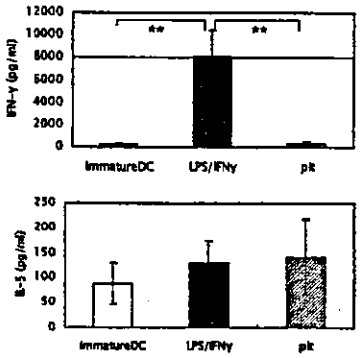

FIGURE 7. Effects of sheared platelets on the cytokine production from naive T lymphocytes. UCB-naive T cells were stimulated with immature DC, LPS- and IFN-γ-treated DC, or DC incubated with sheared platelets for 6 days, and the supernatant of mixed culture wells was examined for cytokine concentrations using a CBA kit. The mean ± SD of 10 experiments are shown, and statistically significant differences are indicated (**, $p < 0.01$).

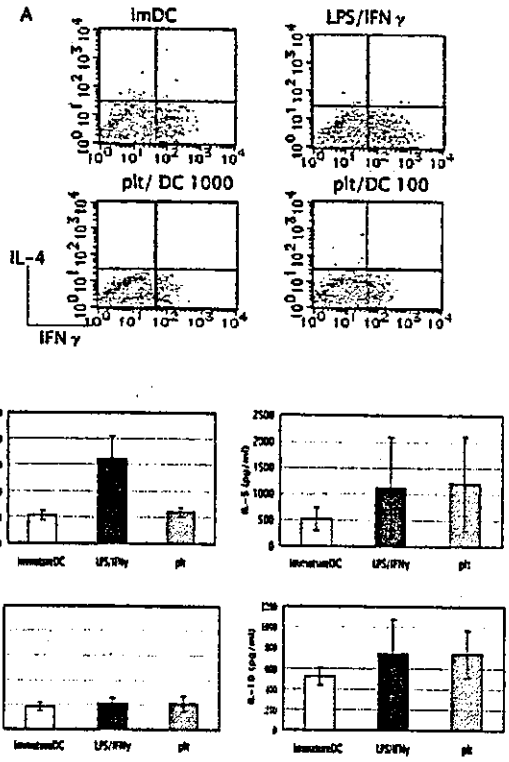

FIGURE 8. Flow cytometric analysis of intracellular cytokine expression (*A*) and measurement of cytokine production (*B*) in allogeneic naive T cells after short term culture with DC and IL-2. Naive T cells were incubated with DC treated as described in Fig. 6 and were further cultured for 4 days in the presence of IL-2 (20 U/ml). *A*, Intracellular expression of IFN-γ and IL-4 was examined by flow cytometry. *B*, Cytokine production was measured using a CBA kit.

The roles of macrophages and T cells in the progression of atherosclerosis and the onset of atheroma rupture have previously been established (1). Many immune-related cells, such as chronically activated $CD4^+$ T cells, exist in atheroma (40). There are several candidate Ags that mediate specific expansion of T lymphocytes, such as viruses or bacteria and endogenous altered Ags, heat shock proteins, or oxidized low density lipoproteins (41). Those Ags, if presented to professional APC such as DC, can guide naive T cells to Th1-type immune effectors. Actually, $CD4^+$ T cell clones in human atherosclerotic lesions specifically respond to oxidized low density lipoproteins in an HLA class II-restricted manner (41). In the present study we focused on DC, which are demonstrated to be in atheroma (10, 11); their role in atherosclerosis was clarified in chronic inflammation related to infection by *Chlamydia pneumoniae* (42), whereas their important roles in immune regulation need to be studied (6). In the case of *Chlamydia* infection, host APCs, especially DC, accumulate at infected endothelial cells, take up bacterial Ags, and produce inflammatory cytokines such as IL-6, TNF-γ, or IL-12/18, which accelerate atherosclerosis (42). IL-12 and IL-18 cause Ag-specific $CD4^+$ T cells to secrete IFN-γ, which is proinflammatory and proatherogenic (7, 8). Platelets accumulate at damaged endothelial cells and are then activated through a gpIb/IX-VWF-mediated interaction, which can result in IL-10-producing DC maturation, as shown in the present study. Those DC may antagonize IFN-γ production and thereby confer atheroprotection. In vitro and in vivo studies have shown that rIL-10 suppresses progressive generation of atherosclerosis (43).

Our study has methodology limitations, particularly in applying the experimental results to understand the role of platelets in regulating local inflammatory responses in vivo. First, we demonstrated DC maturation in the presence of platelets after exposure to high shear only in a pure culture system, excluding the effects of various cellular and protein components in vivo, such as erythrocytes. Other important contributors, such as endothelial cells, extracellular matrix, and complex blood flow conditions, were not considered in our ex vivo system. Second, we demonstrated platelet surface translocation of CD40L and P-selectin only after exposure to homogeneous shear and then could not reproduce the complex shear stress to which platelets are exposed in vivo. Thus, the amounts of CD40L and P-selectin expression may not be physiologic. Although no clinical data regarding platelet surface CD40L are available to date, previous studies have shown P-selectin expression to be induced in a similar manner when platelets pass over the damaged surfaces, i.e., coronary intervention (44). Thus, we believe that platelet activation, with P-selectin and CD40L surface translocation, can be induced by interaction with damaged vascular surfaces under high shear stress conditions in vivo.

In conclusion, it was demonstrated that platelets, after exposure to high shear stress, play an important role in DC maturation and subsequent reactions, including allo-T cell proliferation and cytokine (IL-10) production. Also, the soluble protein factors secreted from these activated platelets contributed to such DC maturation or IL-10 production. Recent in vivo works have shown that platelets were involved in increased risk of atherosclerosis (12, 45). To the contrary, our experimental results suggest that platelets suppress the progression of atherosclerosis through regulating the immunological function of DC. These results show unique, dual functions of platelets and their important roles to maintain equilibrium in the context of local inflammatory responses at sites of vascular injury.

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Ability of Anti-Glycoprotein IIb/IIIa Agents to Dissolve Platelet Thrombi Formed on a Collagen Surface Under Blood Flow Conditions

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OBJECTIVES We examined the lytic effects of anti-glycoprotein (GP) IIb/IIIa agents on platelet thrombi formed on the collagen surface under blood flow conditions.

BACKGROUND Anti-GP IIb/IIIa agents may influence platelet thrombi already formed.

METHODS Blood samples were anticoagulated either by the specific antithrombin Argatroban (100 μM) or by unfractionated heparin (0.1 U/ml). After platelet thrombi were formed on a collagen surface following 6-min perfusion of whole blood obtained from eight adult donors containing fluorescinated platelets at a wall shear rate of 1,500 s^{-1}, additional blood samples from the same donors either containing or not containing anti-GP IIb/IIIa agents (abciximab, eptifibatide, or tirofiban) were perfused on these thrombi. The three-dimensional structures of the platelet thrombi were continuously observed by laser confocal microscopy equipped with a piezo-electric motor control unit and recorded.

RESULTS The platelet thrombi started to dissolve after perfusion of blood containing the anti-GP IIb/IIIa agents, whereas their growth resumed after subsequent perfusion of control blood. Only a single layer of platelets having heights of 3 ± 1 μm, 3 ± 2 μm, and 3 ± 1 μm, respectively, could be seen after 6-min perfusion of blood containing abciximab, eptifibatide, and tirofiban, whereas the initial height of the platelet thrombi of 8 ± 2 μm increased to 11 ± 4 μm after subsequent perfusion of control blood (n = 8). The volume of the platelet thrombi, which was 3,352 ± 1,045 μm^3 before starting the second perfusion, was reduced to 778 ± 102 μm^3, 812 ± 122 μm^3, and 856 ± 144 μm^3 after 6-min perfusion of blood containing abciximab, eptifibatide, and tirofiban, respectively.

CONCLUSIONS We have shown in this study that anti-GP IIb/IIIa agents possess the ability to dissolve platelet thrombi. (J Am Coll Cardiol 2004;44:316–23) © 2004 by the American College of Cardiology Foundation

It is now well known that agents blocking the platelet glycoprotein (GP) IIb/IIIa receptor (integrin $\alpha_{IIb}\beta_3$) reduce the incidence of early thrombotic complications after angioplasty (1–8) and prevent death and myocardial infarction in patients with acute coronary syndromes (8). Anti-glycoprotein IIb/IIIa agents have been reported to inhibit platelet aggregation induced by chemical activation (9,10) or by shearing (11), and to inhibit platelet

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thrombus formation in animal models of endothelial damage (12). In addition to these effects, several previous clinical and animal studies have reported that the anti-GP IIb/IIIa agents enhance the reperfusion rate after administration of fibrinolytic agents and may even induce spontaneous reperfusion (13–18), although the effect was not marked when the dose of fibrinolytic agent was reduced to half (19). These findings led us to wonder whether anti-GP IIb/IIIa agents might also have the potential to augment thrombolysis or induce thrombolysis by themselves, in addition to their well-known preventive effect on thrombus formation.

Previously, platelet aggregation induced by chemical agonists, such as adenosine diphosphate or thrombin, was reported to disaggregate when the binding capacity of GP IIb/IIIa was affected (20,21). However, this experimental finding may or may not be relevant to the question of enhanced thrombolysis induced by these agents in vivo, because the mechanism of platelet thrombus formation in vivo, especially under high shear stress conditions, might not be the same as that underlying platelet aggregation in vitro (22,23). Multiple receptor-ligand interactions, including von Willebrand factor (VWF) binding to both GP Ibα and GP IIb/IIIa, are involved in the former (22,23), whereas the latter is exclusively mediated by fibrinogen binding to activated GP IIb/IIIa (9). In the present study, we found that the anti-GP IIb/IIIa agents available for clinical use, namely, abciximab, eptifibatide, and tirofiban, not only inhibit platelet thrombus formation on a collagen surface under blood flow conditions but also have the potential to dissolve platelet thrombi already formed on the surface. These results may explain why these anti-GP

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Abbreviations and Acronyms
GP = glycoprotein
NIH = National Institutes of Health
VWF = von Willebrand factor

IIb/IIIa agents augment the thrombolytic effects of fibrinolytic agents or induce spontaneous reperfusion by themselves.

METHODS

Sample preparation. The anti-GP IIb/IIIa agents used in this study were abciximab (ReoPro; Centocor, Malvern, Pennsylvania), eptifibatide (Integrellin; Cor Therapeutic, Inc., San Francisco, California) and tirofiban (Aggrastat; Merck & Co., Inc., West Point, Pennsylvania), all of which are available for clinical use in many countries (24). Venous blood from eight normal volunteers abstaining from any type of medication was drawn through 19-G needles into plastic syringes containing one-tenth of their volume of the specific thrombin inhibitor Argatroban (Mitsubishi Kagaku, Tokyo, Japan) (25) or by commonly used anticoagulant of heparin. The final concentrations of the anticoagulant used were 100 μM for Argatroban and 0.1 U/ml for heparin. Argatroban, instead of the more commonly used anticoagulant citrate, was used for anticoagulation, to avoid pleiotropic effects through decreased cation concentration. Heparin, although used commonly in the clinical setting, was used only to show the relevance of the results obtained with Argatroban, because it may influence the results by either activating the platelets or modifying the interaction between VWF and GPIbα (22). Platelets in whole blood were rendered fluorescent by the addition of mepacrine (Sigma Co. Ltd., St. Louis, Missouri), according to a previously established procedure (25).

Preparation of the flow chamber and visualization of the platelet thrombi. Acid-insoluble fibrillar type I collagen from bovine Achilles tendon (Sigma Co.) was immobilized on a glass coverslip (Corning Inc., Acton, Massachusetts; 24 mm × 50 mm) in a parallel-plate flow chamber (25). The distance between the two glass plates was fixed at 220 μm by the placement of a silicon gasket. Then, the blood samples were introduced into the chamber with a syringe pump (Harvard Apparatus Co. Ltd., Holliston, Massachusetts) at a constant flow rate to achieve a wall shear rate of 1,500 s^{-1}. Platelet thrombi forming on the surface of collagen were visualized with an inverted stage epi-fluorescence video-microscope system equipped with a 480-nm excitation light source (DM IRB, 1RB-FLUO; Leica, Wetzlar, Germany) (25). The microscopic images were digitized online with a photosensitive color charge-coupled device camera (L-600; Leica) and stored as digital images in a personal computer (Power Macintosh G4; Apple Co. Ltd., Palo Alto, California). To quantify the percentage surface area coverage by the platelets, the digital color images were converted into black-and-white images using the National Institutes of Health (NIH) Image software (public domain software by Dr. Wayne Rasband, NIH, version 1.62), and the percentage surface area coverage by the platelets was calculated.

To detect the effects of anti-GP IIb/IIIa agents of dissolving platelet thrombi formed on the collagen surface, three-dimensional structural analysis was conducted using an ultra-fast laser confocal microscope equipped with a piezo-electric motor control unit (Fig. 1A). Using a confocal unit composed of a rapidly rotating disk having 20,000 pinholes and micro lenses on it (CSU10; Yokogawa Medical Co., Tokyo, Japan), each confocal image could be obtained within 10 ms (26). To visualize the three-dimensional structure of the platelet thrombi formed on the collagen surface, the objective lenses were up and down (20 μm/50 s) at a constant speed controlled by a piezo-electric motor control system, so that scanning images of the thrombi were obtained. The confocal images were enhanced using an image intensifier (SRUB GEN III+, Solamer, Salt Lake City, Utah, and Intermedical Co., Tokyo, Japan), and the intensified images were stored in a digital video recorder (HandyCum; Sony Co., Tokyo, Japan) and transferred to a personal computer (Power Macintosh G4, Apple Co. Ltd.). Three-dimensional projection images of the thrombi were obtained using shareware NIH images, as previously reported (26). For quantification, the cross-sectional area covered by platelets was calculated at the base and 3, 6, and 9 μm above the base of the platelet thrombi. The results were expressed as a percentage of the area covered at the base of the thrombi. The volume of the platelet thrombi was calculated by z-section integration of the cross-sectional area in each micrometer.

Experimental protocol. Experiments were performed as described in Figure 1B and its legend. Briefly, 15 ml of blood samples either containing or not containing anti-GP IIb/IIIa agents at concentrations sufficient enough to inhibit platelet thrombus formation on the collagen surface (abciximab: 10 μg/ml; eptifibatide and tirofiban: 0.5 μM) was perfused on the same collagen surface on which platelet thrombi were already formed by the initial 15 ml of control blood perfusion. The second perfusion, of blood either containing or not containing the anti-GP IIb/IIIa agents, was started immediately after the first perfusion was completed, without stopping the blood flow, to avoid the falling of leukocytes onto the platelet thrombi because of gravity, as it is speculated that the presence of leukocytes may influence the stability of the platelet thrombi. The three-dimensional structures of the platelet thrombi were assessed every 50 s by the three-dimensional imaging technique described earlier. All the experiments were performed at room temperature, controlled between 22°C to 26°C.

Statistical analysis. All numerical results were presented as mean ± SD, unless otherwise stated. The effects of various concentrations of the three anti-GP IIb/IIIa agents under study on the percentage surface area coverage by the

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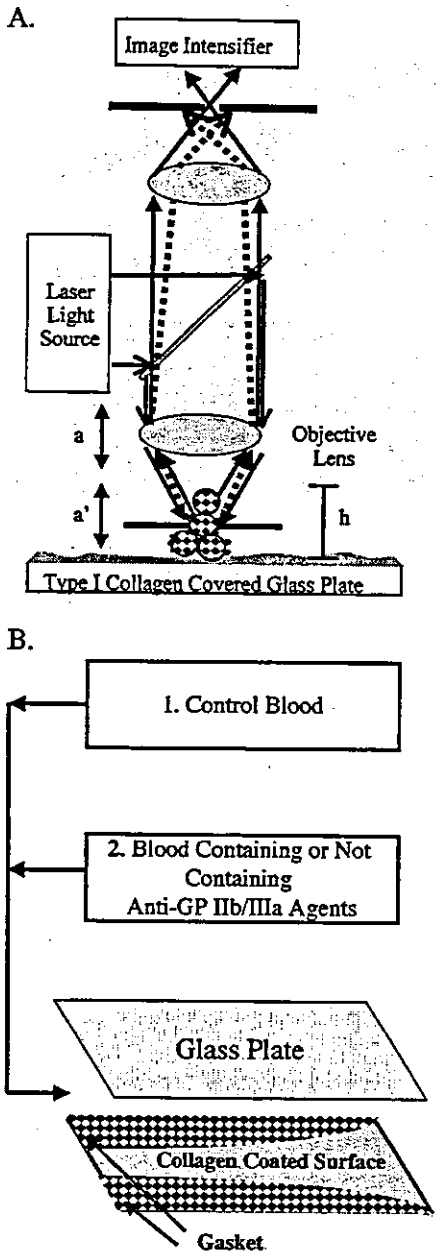

Figure 1. Experimental protocol. Fifteen ml of blood anticoagulated either by Argatroban or heparin containing platelets rendered fluorescent by the addition of mepacrine was perfused in a parallel-plate flow chamber composed of two glass plates, one of which was covered by immobilized type I collagen. A perfusion of an additional 15 ml of blood obtained from the same donors and treated by the same procedure, containing or not containing one of the anti-glycoprotein (GP) IIb/IIIa agents, was immediately started to perfuse on the same collagen surface for the same length of time (B). The two-dimensional and three-dimensional structures of the platelet thrombi formed on the collagen surface were continually assessed by fluorescence microscopy or by a laser confocal microscope controlled by a piezo-electric motor control system (A). In a laser confocal imaging system, the platelet thrombi were scanned from the bottom to the top (a') at a constant speed by controlling the position of objective lens (a) by piezo-motor control unit. Then, the scanning confocal images were projected from the top to the bottom at every 5° to obtain three-dimensional projection images, including projections from the top, 45° position from the horizontal axis and the side of the platelet thrombi, which are shown in Figures 3 and 4. The maximum height of the platelet thrombi (h) was calculated based on the projection image from the side of the platelet thrombi.

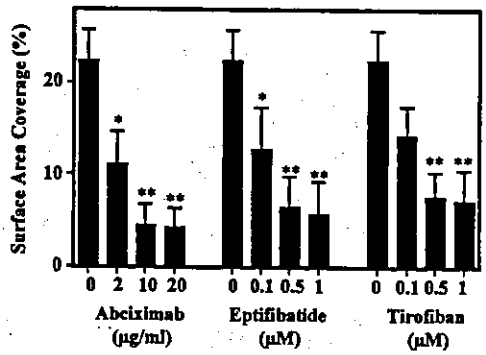

Figure 2. Effects of anti-glycoprotein (GP) IIb/IIIa agents on platelet thrombus formation on the collagen surface under blood flow conditions. Fifteen ml of blood containing fluorescent platelets were perfused on the collagen surface for 6 min, either in the presence or absence of the anti-GP IIb/IIIa agents abciximab, eptifibatide, and tirofiban at the final concentrations shown in the figure. The results shown represent the mean and standard deviation of the eight sets of replicated experiments. The * and ** indicate values significantly lower than those in the absence of the anti-GP IIb/IIIa agents with the p value <0.05 and <0.01, respectively.

platelets were tested by two-way analysis of variance. The effects of the second perfusion of blood containing one of the various anti-GP IIb/IIIa agents on the volume and maximum height of the platelet thrombi already formed were also tested by one-way analysis of variance. The differences between groups of data were assessed by Newman-Keuls test. A p value of <0.05 was considered to denote statistical significance.

RESULTS

Effect of anti-GP IIb/IIIa agents on platelet thrombus formation on the collagen surface. All the anti-GP IIb/IIIa agents tested in our study inhibited platelet thrombus formation on the collagen surface in a dose-dependent manner no matter whether blood was anticoagulated with Argatroban or heparin (Fig. 2). There were no statistically significant differences between the effects of abciximab and eptifibatide, or between those of eptifibatide and tirofiban. On the other hand, the percentage surface area coverage of 7.7 ± 2.6% achieved with tirofiban at a dose yielding the maximum inhibitory effect (0.5 μM) was significantly higher than that of 4.6 ± 2.2% achieved with the maximum inhibitory dose of abciximab (10 μg/ml; $p < 0.05$). Only one layer of attached platelets, without z-section growth mediated by platelet cohesion, was observed after perfusion of blood containing abciximab (10 μg/ml), eptifibatide (0.5 μM), or tirofiban (0.5 μM), whereas three-dimensional growth mediated by platelet cohesion was observed in the absence of these agents (Fig. 3).

Effect of anti-GP IIb/IIIa agents on platelet thrombi formed on the collagen surface. Soon after blood containing any one of the anti-GP IIb/IIIa agents began to be perfused over the collagen surface where platelet thrombi had formed, the thrombi started to dissolve no matter whether blood was anticoagulated by Argatroban or heparin. On the other hand, the platelet thrombi continued to grow after subsequent perfusion of blood not containing any of the

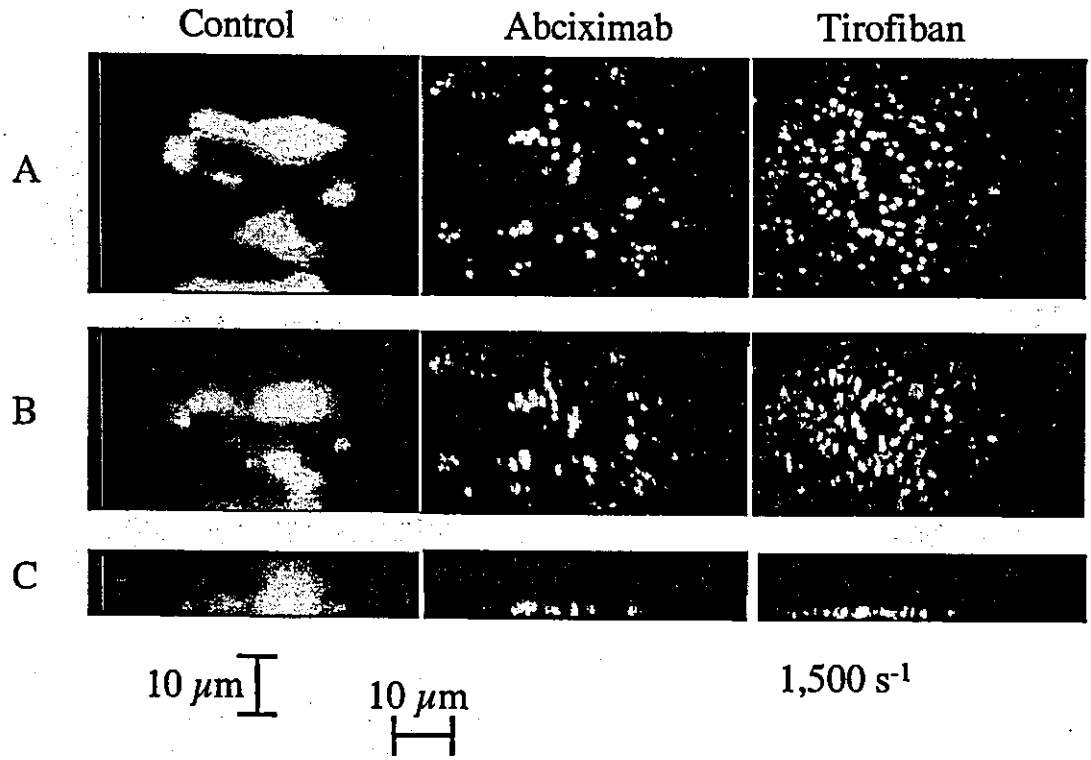

Figure 3. Effects of anti-glycoprotein (GP) IIb/IIIa agents on three-dimensional platelet thrombus growth on the collagen surface. Experiments were performed as described in the legend for Figure 2, but the platelet thrombi were visualized by laser confocal microscopy. The projection images from the top **(A)**, 45° position from the horizontal axis **(B)**, and the side **(C)** are shown. Three-dimensional platelet thrombus growth occurred in the control group **(left panel)**, whereas only a single layer of attached platelets was seen in the presence of any of the anti-GP IIb/IIIa agents tested (10 μg/ml abciximab, 0.5 μM eptifibatide, and tirofiban). The results shown are representative of the results of eight replicated experiments. The results with eptifibatide were similar to those obtained with abciximab and tirofiban, but are not shown because of space limitation. There were no differences in the results when the blood was anticoagulated by heparin. For the accompanying videos corresponding to Figure 3 (Videos 1, 2, and 3), please see the July 21 issue of *JACC* at www.cardiosource.com/jacc.html.

anti-GP IIb/IIIa agents. Not only single platelets, but also masses of platelet thrombi became detached from the body of the thrombi when blood containing an anti-GP IIb/IIIa agent was perfused. Three-dimensional analysis of the thrombi revealed that both the height and the volume of the platelet thrombi began to decrease owing to dissolution of platelet cohesion (Figs. 4 and 5). The maximum height as well as the volume of the platelet thrombi immediately before and after completion of perfusion of blood containing one of the anti-GP IIb/IIIa agents, abciximab (10 μg/ml), eptifibatide (0.5 μM), or tirofiban (0.5 μM), are summarized in Table 1. Indeed, the maximum height of the thrombi and their volume decreased significantly after 6-min perfusion of blood containing one of the anti-GP IIb/IIIa agents.

Three-dimensional imaging revealed only a single layer of platelets remained after 6-min perfusion of blood containing one of the anti-GP IIb/IIIa agents (Fig. 5). There were no differences in the effects on the platelet thrombi among abciximab, eptifibatide, and tirofiban.

DISCUSSION

Anti-GP IIb/IIIa agents were developed as antiplatelet agents, based on the premise that they would block platelet aggregation (7–9). Clinical experiences, as well as animal experiments, have demonstrated that early reperfusion of coronary arteries can be induced by the administration of anti-GP IIb/IIIa agents, regardless of whether or not fibrinolytic agents were also administered concomitantly (13–19). We showed that the widely used GP IIb/IIIa antagonists abciximab, eptifibatide, and tirofiban have the potential to dissolve platelet thrombi formed on a collagen surface. We postulate that the effect of these agents of dissolving platelet thrombi already formed, in addition to their preventive effect on de novo platelet thrombus formation, contributes to the reportedly augmented thrombolytic effects observed when these agents are administered in combination with fibrinolytic agents, and to the increased rate of reperfusion achieved when these agents are used alone (13–19).

Unlike in the case of platelet aggregation induced by chemical agonists, which is known to be mediated exclusively by fibrinogen binding to activated GP IIb/IIIa, platelet thrombus formation on the collagen surface require stimulation of several platelet receptors induced by their binding of the corresponding ligands, including the GP Ibα-VWF interaction (27–29), GP IIb/IIIa ligation by plasma proteins including fibrinogen and VWF (27–29), $P2Y_1$ and $P2Y_{12}$ stimulation by adenosine diphosphate released from activated platelets (30,31), are involved in

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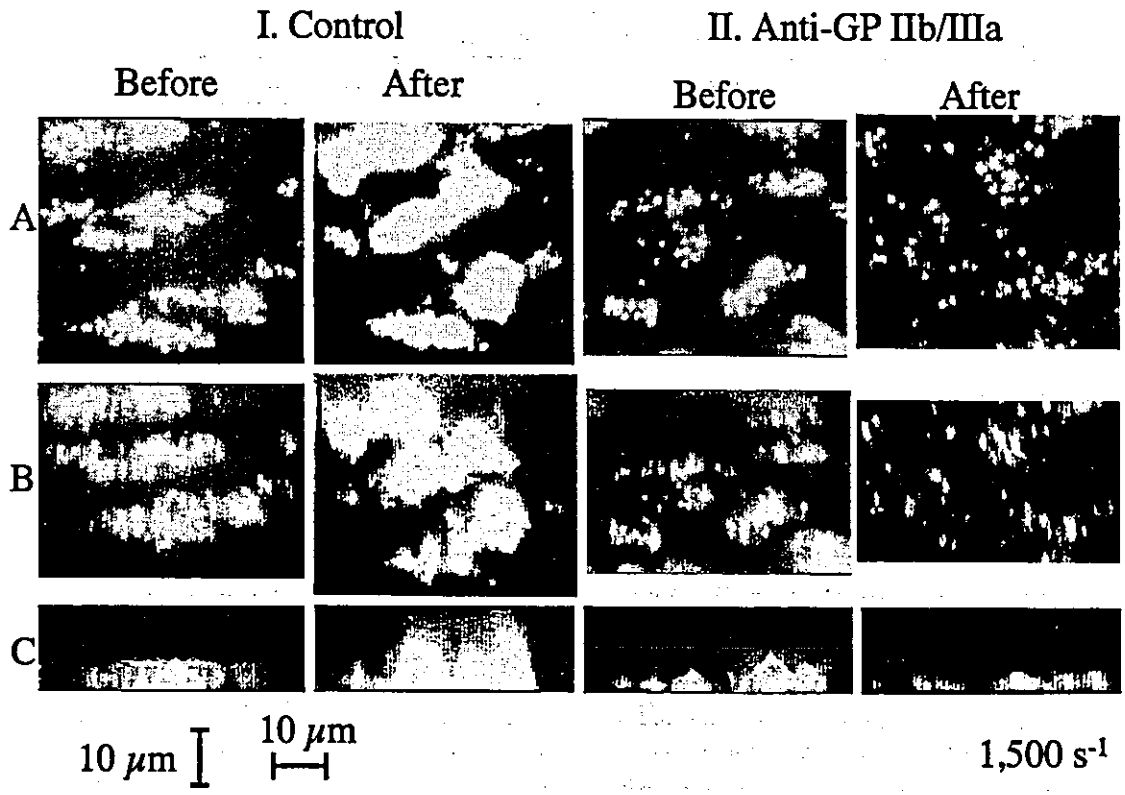

Figure 4. Effect of anti-glycoprotein (GP) IIb/IIIa agents on the dissolution of platelet thrombi formed on the collagen surface. The experiments were performed as described in the legend for Figure 1B. Blood anticoagulated with Argatroban either containing or not containing anti-GP IIb/IIIa (abciximab: 10 μg/ml, eptifibatide: 0.5 μM, tirofiban 0.5 μM) was perfused on the collagen surface on which platelet thrombi had already formed as a result of previous perfusion of control blood. The three-dimensional projection images of the platelet thrombi (the projection images from the top **[A]**, 45° position from the x axis **[B]**, and the side **[C]**) before and after the second perfusion of blood containing (II) or not containing one of the anti-GP IIb/IIIa agents of abciximab (I) are shown in the **left and right panel** of I and II. The results are representative of those of eight replicated experiments. No differences in effects were observed among the three anti-GP IIb/IIIa agents tested. No differences in the effects of anti-GP IIb/IIIa agents were observed when blood anticoagulated with heparin was used. For the accompanying videos corresponding to Figure 4 (Videos 4, 5, 6, and 7), please see the July 21 issue of *JACC* at www.cardiosource.com/jacc.html.

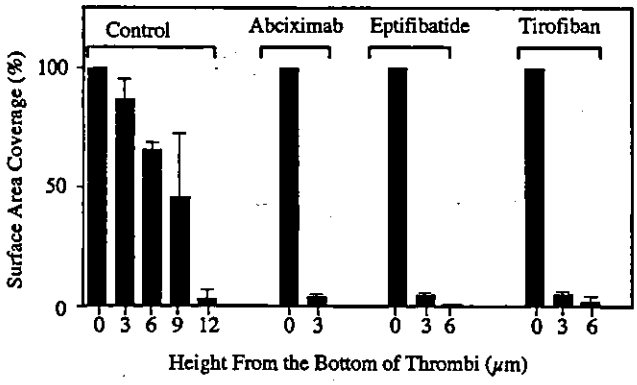

Figure 5. Effect of anti-glycoprotein (GP) IIb/IIIa agents on platelet thrombi formed on the collagen surface. The experiments were performed in a manner similar to that described in the legend for Figures 1 and 4. The three-dimensional structure of the platelet thrombi formed on the collagen surface after 6-min perfusion of blood on the collagen surface at 1,500 s^{-1} was quantified by calculating the cross-sectional areas of the platelet thrombi using the National Institutes of Health image software at every 3 μm from the collagen surface. The results are expressed as a percentage of the largest cross-sectional area obtained at the collagen surface level. The results shown are the mean and standard error of eight replicated experiments. Similar results were obtained in the condition when blood was anticoagulated by heparin.

platelet cohesion under high shear stress conditions. Although numerous receptor-ligand interactions, such as the initial tethering mediated by VWF-GP Ibα interaction, may play a role in the formation of platelet thrombi, our present findings suggest that GP IIb/IIIa ligation is required for stable platelet cohesion because only platelet bound with platelet, but not with the matrix surface was susceptible to the second perfusion of blood containing one of the anti-GP IIb/IIIa agents. Further studies using our assay system, including the clarification of the relative importance of other factors, such as CD40 ligand (32) and P-selectin (33), are under way.

Possible mechanism. We propose the following mechanism to explain how platelet thrombi are dissolved by anti-GP IIb/IIIa agents (Fig. 6); however, please note that the mechanism shown in Figure 6 is purely speculative. First, we speculate that the binding of crucial ligands, whether VWF or fibrinogen, is reversible; therefore, ligand molecules can be replaced by anti-GP IIb/IIIa agents when thrombi are perfused with blood containing these agents. Platelets begin to get detached from the thrombi when the

Table 1. Effects of Subsequent Perfusion of Blood Containing One of the Anti-GP IIb/IIIa Agents on the Height and the Volume of the Pre-Existing Platelet Thrombi*

	Before the Second Perfusion†	After the Second Perfusion§			
		Control‡	Abciximab	Eptifibatide	Tirofiban
Height of thrombi (μm)	8 ± 2	15 ± 4‖	3 ± 1‖	3 ± 2‖	3 ± 1‖
Volume of thrombi (μm³)	3,352 ± 1,045	7,450 ± 1,825‖	778 ± 102‖	812 ± 122‖	856 ± 144‖

*The height and the volume of the platelet thrombi before and after the second perfusion of blood containing or not containing abciximab (10 μg/ml), eptifibatide (0.5 μM), or tirofiban (0.5 μM) are shown. †Values immediately before the commencement of the second perfusion. ‡After completion of the second perfusion of blood not containing any of the anti-GP IIb/IIIa agents. §Values after completion of the second perfusion of blood containing any one of abciximab, eptifibatide, or tirofiban. ‖Indicates that the difference compared with the value determined before the second perfusion was significant.
GP = glycoprotein.

crucial number of ligand molecules necessary to maintain the integrity of the thrombi is replaced by anti-GP IIb/IIIa agents, and the power generated by the binding of ligands to GP IIb/IIIa is no longer sufficient to resist the shear force generated by blood flow. Other adhesive proteins, including P-selectin (33) and CD40 ligand (32), may be involved in stabilizing platelet thrombi, but their roles were not investigated in the present study.

Advantages and limitation of our methods. Our newly developed three-dimensional analysis system depended on the unique features of the confocal microscopy system (26). However, there were still obvious methodological limitations in our assay system, especially when seeking to apply the experimental results to understand the events in vivo. Obviously, we could not reproduce the complex in vivo arterial flow conditions (34) in our flow chamber system. Under the complex flow conditions prevailing in vivo, including low, high, or changing shear rate conditions, the ability of anti-GP IIb/IIIa agents to dissolve platelet thrombi might be greater or lesser than that shown in our experiments. We also conducted the experiments at room temperature rather than at body temperature (37°C), mostly because the thrombi were more prominent, and the volume of blood necessary for thrombus formation was smaller at room temperature (28). Nevertheless, the important message that anti-GP IIb/IIIa agents can cause dissolution of platelet thrombi by adversely affecting platelet cohesion is not likely to be influenced by these methodological limitations.

One might argue that the ability of anti-GP IIb/IIIa agents to dissolve platelet thrombi in vivo might be weaker than that shown in our experiment, because fibrin formation, which plays a crucial role in stabilizing thrombi, was inhibited under our study conditions. The mass of platelet thrombi detached from the body of the platelet thrombi we have detected herein, might also be unstable without fibrin

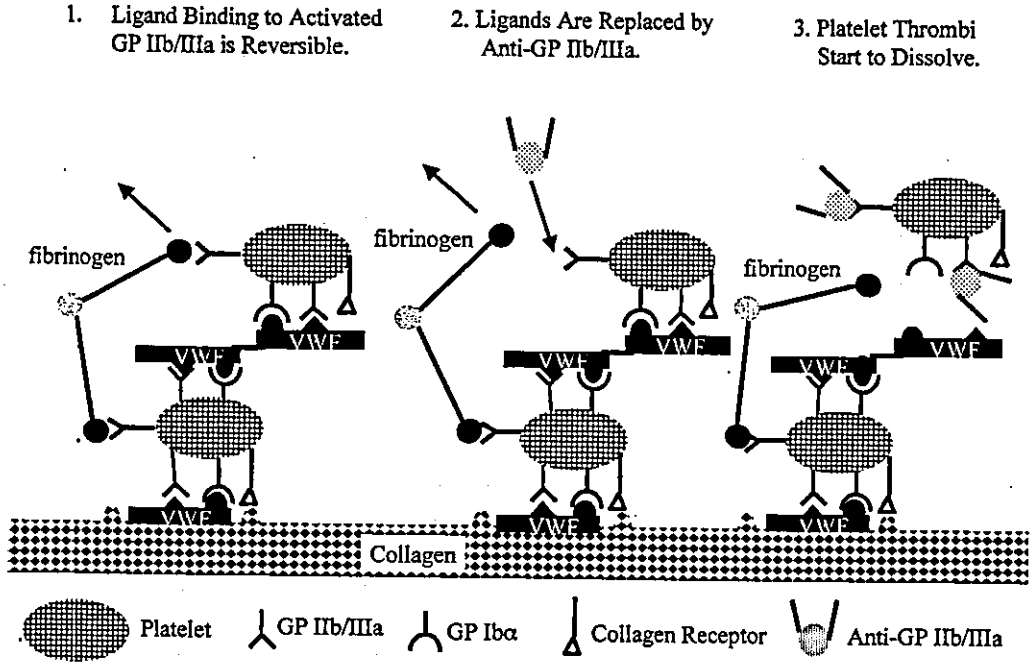

Figure 6. Speculative mechanism explaining the thrombus-dissolving effects of anti-glycoprotein (GP) IIb/IIIa agents. This figure summarizes the possible mechanism by which anti-GP IIb/IIIa agents dissolve platelet thrombi formed on a collagen surface, although it is not clear whether von Willebrand factor (VWF), in addition to fibrinogen, also plays some role in stabilizing platelet thrombi. When blood containing one of the anti-GP IIb/IIIa agents began to be perfused, ligand bound with activated GP IIb/IIIa was replaced by the anti-GP IIb/IIIa agent. Parts of the platelet thrombi started to become detached when a certain number of ligands were replaced by anti-GP IIb/IIIa and the strength of the GP IIb/IIIa ligation was no longer sufficient to support the integrity of the thrombi. Details are explained in the "Discussion" section.

and may dissociate into small aggregates incapable of embolization. Nonetheless, our experimental results might have in vivo relevance because anti-GP IIb/IIIa agents tend to be used in combination with anticoagulant and thrombolytic therapy, which dissolve fibrin net. Indeed, we have shown that the dissolution effects of anti-GP IIb/IIIa agents could still be seen when blood was anticoagulated by heparin at concentration achieved with clinical use.

Another important issue we could not completely address in this paper was the effects of time on the stability of platelet thrombi. Indeed, we could not demonstrate whether the dissolution effects we described herein still occurred when the subsequent perfusion of blood containing anti-GP IIb/IIIa agents was started hours after the formation of platelet thrombi. This issue is relevant because the binding of fibrinogen to activated GP IIb/IIIa was reported to be reversible initially and became irreversible later (35). We could not conduct experiments to answer this question directly, because continuous perfusion, which prevents the falling of leukocytes onto the platelet thrombi because of gravity, was necessary to ensure exclusion of the possible effects of leukocytes on the stability of the platelet thrombi. The dissolution effects of anti-GP IIb/IIIa agents, although not exactly demonstrated as described in this study, could be seen even when the subsequent perfusion of blood containing anti-GP IIb/IIIa agents started to be perfused after 30 min from the end of initial blood perfusion (data not shown).

Conclusions. We have previously shown that different anti-GP IIb/IIIa agents might have different effects on platelet activation under high shear stress condition (36,37). Although there is still ongoing debate on whether or not different anti-GP IIb/IIIa agents have different antithrombotic effects in vivo (24,38), it would be reasonable to suppose that all three anti-GP IIb/IIIa agents have similar effects on thrombus dissolution when used in doses required to block platelet aggregation. In conclusion, we have demonstrated the dissolution effects of various anti-GP IIb/IIIa agents on platelet thrombi formed on the collagen surface under blood flow conditions.

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